Cell, Vol. 114, 401–404, August 22, 2003, Copyright ©2003 by Cell Press

In the Beginning: Lessons from Fertilization in Mice and Worms

Minireview

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Sexual reproduction proceeds by fertilization; formation of new individuals by the union of haploid gametes. Recent reports in *Cell* and in *Developmental Cell* may provide new insights as to how this process begins and is regulated.

Mammalian fertilization is a precisely coordinated series of cellular interactions in which sperm recognize and contact eggs, gametes fuse, and the egg is activated. Models of these events shape our thinking about the initiation of development and guide new approaches to infertility treatment and contraceptive design.

The Current Model: Strengths and Gaps

Gamete interaction begins in mammals when sperm bind to the egg extracellular coat, or zona pellucida (ZP), in the ampulla of the oviduct. The mechanism by which sperm interact with the ZP is articulated in a working model of the early events of fertilization. This model consists of core features that are widely accepted at present as well as additional features which are still the subject of controversy. Those core features may be summarized as follows (see Figure 1; for details and references, see Wassarman et al., 2001; Primakoff and Myles, 2002; Evans and Florman, 2002).

Step 1. The mouse ZP is constructed from three glycoproteins (ZP1, ZP2, and ZP3), of which ZP3 retains the anticipated characteristics of a sperm adhesion molecule following purification: it binds directly to sperm and specifically to the region of sperm known to interact with the ZP, and it is a competitive inhibitor of sperm-ZP adhesion in vitro (Figure 1). The sperm adhesion domain on ZP3 is believed to be a glycan and a candidate O-linked oligosaccharide has been partially purified. However, there is no consensus regarding the sequence of the sperm adhesion glycan or the molecular nature of the glycan binding protein on sperm.

Step 2. Bound sperm are activated and undergo a secretory event, the acrosome reaction (AR). Purified ZP3 completely accounts for the AR-inducing activity of the ZP in in vitro assays whereas purified ZP1 and ZP2 lack this activity (Figure 1).

Step 3. Sperm are believed to establish secondary interactions with ZP2 after completion of the AR and then penetrate the ZP (Figure 1).

Step 4. Sperm contact and fuse with the egg plasma membrane. The egg tetraspanin, CD9, is required during this process, however other protein components of the adhesion/fusion machinery are not known.

Step 5. Successful embryonic cleavage and development occurs only when eggs are fertilized by a single sperm (Figure 1). This is assured in most mammalian species by a block to polyspermy that occurs at the egg plasma membrane and prevents fusion of additional sperm. The ZP is also modified following fertilization so that sperm binding sites and AR-inducing activity are eliminated and the ZP matrix becomes refractory to sperm penetration. Sperm-egg fusion leads to the secretion of egg cortical granule contents onto the ZP. These contents are believed to include a protease which clips ZP2 and accounts for the decreased penetrability of the matrix. In addition, ZP3 from 2-cell embryos has apparently been inactivated as the purified glycoprotein lacks the characteristics of a sperm adhesion molecule in vitro and will no longer induce the AR. The standard model proposes that this is due to the action of an unidentified glycosidase or protease activity that is released from cortical granules and inactivates sperm adhesion glycans on ZP3.

Do the core elements of this model need to be extended or recast in light of several new reports?

How Do Sperm Associate with the ZP?

According to the current model, sperm adhere to the ZP by associating with O-linked glycans on ZP3; secondary interactions with ZP3 then trigger the AR (Wassarman et al., 2001; Primakoff and Myles, 2002; Evans and Florman, 2002). Within this context we now consider SED1, a mouse homolog of procine sperm p47 protein. Sperm binding to the ZP is inhibited by SED1 antibodies and by recombinant SED1, presumably by blocking binding sites on sperm and ZP, respectively. In addition, sperm of mice with targeted deletions of the *SED1* gene exhibit reduced binding to ZP in vitro and the males have somewhat reduced fertility (Ensslin and Shur, 2003).

How does SED1 mediate gamete binding? According to the current model, sperm-ZP adhesion is mediated by ZP3 and other ZP glycoproteins do not participate in this process, yet SED1 interacts with both ZP2 and ZP3 in in vitro binding assays (Ensslin and Shur, 2003). This specificity suggests that it is involved in a stage of gamete interaction that had not previously been described. One possibility is that it acts at an earlier stage to tether highly motile sperm to the ZP prior to the more specific interactions mediated by ZP3. In vitro studies show that sperm may reside at the ZP surface for a single flagellar beat, as little as 50 ms, before swimming away, and the first steps of adhesion must occur during this period. The current model requires that sperm identify a subset of ZP3 glycans along the repeating ZP2/ZP3 filaments that comprise the ZP within this time frame. SED1 could provide an initial process in which sperm interact with either of the proteins along the filament to form first contact. This may be followed by specific interactions with ZP3 that reflect either a later stage of adhesion or the activation events associated with the AR. In fact, low and high affinity binding sites for ZP3 were identified on sperm by direct binding studies (Thaler and Cardullo, 1996) and SED1 may provide one of these binding sites. This is reminiscent of lymphocyte rolling and arrest. Lymphocytes are slowed during

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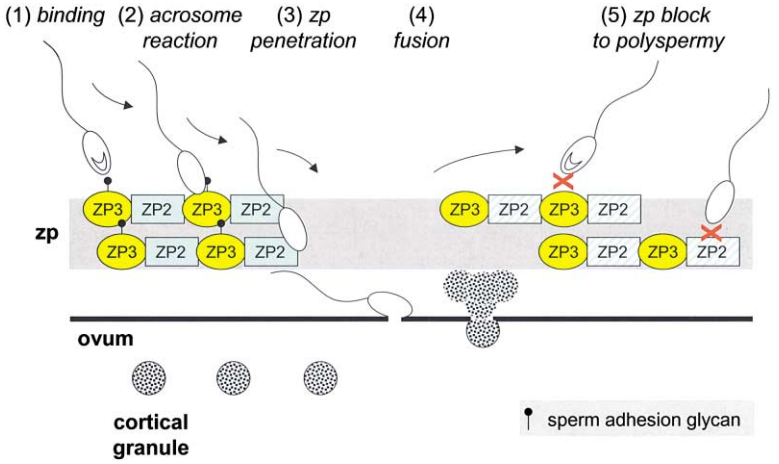

Figure 1. The Current Model of Mammalian Sperm-Egg Interaction and the Impact of Several New Reports

(1) Sperm bind initially to a glycan on ZP3, although data from Ensslin and Shur (2003) is consistent with the presence of an earlier step mediated by SED1. (2) ZP3 then triggers the AR. The work of Xu and Sternberg (2003) may provide new insights into this signal transduction pathway. Acrosome-reacted sperm (3) form secondary contact with ZP2 during ZP penetration and then (4) fuse with eggs. (5) A ZP block to polyspermy sets in after fusion due to release of egg cortical granule contents. The current model assigns local roles to a ZP3-inactivating activity and a ZP2 protease (red blocks) whereas Rankin et al. (2003) suggest a novel new mechanism (see text for details).

movement through the bloodstream by selectin interaction with endothelial glycans and this permits a second phase of adhesion that is mediated by integrins and leads to extravasation (Springer, 1994).

Yet if SED1 mediates an obligatory preliminary stage of gamete interaction then we might expect a more profound decrease in the fertility of mice in which the gene is disrupted. Future studies must define the role of SED1 within the sequence of events of gamete interaction.

How Do Sperm Fuse with Eggs?

After penetrating the ZP, sperm bind to and fuse with egg plasma membrane. Until recently, it was believed that mammalian gamete membrane adhesion was mediated by sperm ADAM proteins associating with egg integrins. However, a series of targeted gene deletion studies now cast doubts on a role of these proteins (He et al., 2003). New insights may be available from an unexpected source: fertilization in *C. elegans* (Xu and Sternberg, 2003). Nematode eggs do not have a coat which presents a barrier to sperm attachment and so differ from mammalian eggs encased in a ZP. Similarly, nematode sperm lack acrosomes or a requirement for the AR (Singson, 2001). This potentially simplifies the analysis of gamete interactions.

C. elegans sperm contain TRP3, a nematode member of the TRPC (transient receptor potential canonical) family of Ca^{2+}-conducting cation channels. Three TRP genes are present in *C. elegans* (Minke and Cook, 2002). but a role for these channels in gamete function had not been reported. In mammals, TRPC channels are present in sperm where they participate in induction of the acrosome reaction by ZP3 (Jungnickel et al., 2001) and may also mediate other functions. Xu and Sternberg (2003) report that *C. elegans* lacking a functional *trp-3* gene are infertile and their sperm, despite being motile and able to bind to the egg, cannot fuse with the egg.

How can TRP3 control fertilization? TRP3 is a functional Ca^{2+} channel, as shown in heterologous expression studies and by analysis of pharmacologically evoked Ca^{2+} responses in sperm from nematodes with a deleted *trp-3* gene as compared to those from wild-type animals (Xu and Sternberg, 2003). A Ca^{2+}-based mechanism is plausible since cell-cell fusion is regulated by elevations of intracellular Ca^{2+} (Shemer and Podbilewicz, 2003). However, TRPC cation channels also conduct an inward Na^{+} current and depolarize membrane potential under physiological conditions (Minke and Cook, 2002). Further work is certainly required to demonstrate that the effects in nematode sperm are due to Ca^{2+} entry.

How is TRP3 activated? This issue, although not addressed by Xu and Sternberg, may provide an unanticipated opportunity to address key questions in fertilization. TRPC channels are thought to be activated through a phospholipase C- (PLC-) dependent mechanism (Minke and Cook, 2002). If *C. elegans* sperm TRP3 is activated during interaction with eggs, as suggested by Xu and Sternberg (2003), and if, as expected, this is a receptor/PLC-dependent mechanism, then what is the receptor and what are the molecular targets of this pathway coupling TRP3 function to fusion?

Little is known about the cellular events that precede nematode gamete fusion (Singson, 2001) and it is necessary to determine whether TRP3 directly regulates cell fusion or acts at a more proximal step. In this regard, mammalian sperm are also activated during the initial contact with egg-derived proteins, which takes place at the ZP. This is mediated by ZP3, which stimulates PLC (Fukami et al., 2003) and leads to Ca^{2+} entry through a TRPC ion channel and acrosomal exocytosis (Jungnickel et al., 2001). The receptor that drives this signal transduction process is unidentified. There is no apparent exocytotic step equivalent to the AR during sperm-egg interaction in *C. elegans*. If a homolgous signal transduction pathway is present in nematodes and mammals, then analysis of the upstream regulators of TRP3 in *C. elegans* sperm may provide insights into the control of the mammalian sperm AR.

How Does the ZP Block Polyspermic Fertilization?

According to the current model, the ZP block to polyspermy is produced by the concerted actions of two cortical granule activities: a ZP3-inactivating factor that modifies or releases sperm adhesion glycans; and a protease that cleaves ZP2 and converts the ZP into a matrix that can no longer be penetrated by sperm. The presence of a ZP3-inactivating activity has been detected indirectly but not been characterized yet. In contrast, proteolytic cleavage of ZP2 is readily apparent following fertilization and its time course roughly corresponds to the onset of the ZP block. A ZP2 protease

is released from activated mouse eggs and has been partially characterized. (Wassarman et al., 2001). These aspects of the current model are consistent with much of the available data, yet it has been difficult to devise critical tests of the model or to determine the mechanism by which ZP2 proteolysis leads to functional alteration of the ZP.

A new opportunity to readdress these issues may be provided by a recent analysis by Rankin et al. (2003) of transgenic mice in which the mouse *zp2* gene was disrupted and replaced with human *zp2* or in which mouse *zp2* and *zp3* were replaced by human transgenes (referred to as ZP2- or ZP2/ZP3-rescue mice). These mice are fertile and females produce eggs that have a ZP, release cortical granule contents after fertilization, and mount a block to polyspermy. However, there are several interesting aspects of the phenotypes of these rescue animals.

Prior to fertilization in vitro, rescue mice with ZP that contain human ZP3 and not mouse ZP3 bind mouse sperm and not human sperm in vitro. The maintenance of taxon selective sperm adhesion is predicted by the current model of fertilization which attributes adhesion activity to ZP3 glycans, as the glycosyltransferase machinery of mouse eggs is expected to assemble glycans onto human ZP3 that are similar (though perhaps not identical) to those on mouse ZP3.

After fertilization in vitro sperm remain associated with the ZP of ZP2- and ZP2/ZP3-rescue mice, unlike wild-type mice where sperm are not found on the ZP after fertilization. In addition, ZP of rescue mouse eggs exhibit little, if any, proteolytic cleavage of ZP2 and yet produce an effective ZP block to polyspermy (Rankin et al., 2003). Rankin et al. suggest that these results may not be consistent with the current model of fertilization. Specifically, they argue that a ZP3-inactivating enzyme released by cortical granules should have similar access to its substrate in both ZP2-rescue and wild-type mice. The persistence of sperm at the ZP of rescue mice after fertilization implies to them that primary sperm adhesion sites have not been inactivated in these animals. Since the current model asserts that such primary sites are provided by ZP3, then it would follow that ZP3 is not necessarily related to sperm adhesion and its regulation.

An alternative model is forwarded in which fertilization is controlled by the higher ordered structure of the ZP. Specifically, they propose that sperm access to binding sites (which remain undefined) is determined not by the properties of an individual glycoprotein such as ZP3, but by the structure of the ZP matrix. Further, it is proposed that the conformation of the ZP matrix is altered after fertilization by ZP2 proteolysis and this prohibits sperm access to ZP adhesion sites. Since human ZP2 is not proteolytically cleaved after gamete fusion in rescue mice, sperm binding sites remain available and binding continues (Rankin et al., 2003).

Can the current model account for these aspects of the transgenic rescue phenotype? It can accommodate persistent sperm association with the embryo ZP in rescue mice, although the current model and the new supermolecular structure model make strong predictions that should readily allow these formulations to be differentiated. Rankin et al. would predict that fresh sperm that are incubated with embryo ZP of rescue mice would continue to bind since adhesion sites remain available in the absence of ZP2 proteolysis; and that these sperm have intact acrosomes, consistent with many studies showing that sperm initially associate with the ZP by means of plasma membranes overlying the acrosome and then initiate the AR. In contrast, the current model would make two predictions. First, it predicts that there would be no fresh binding of acrosome intact sperm to the ZP of rescue mouse embryos. Second, it requires that the sperm that do not fall off the embryo ZP of rescue mice must all have completed the AR and be within the ZP during transit. In fact, the current model suggests that sperm make secondary associations with ZP2 after completing the AR: since the rescue animals exhibit no detectable ZP2 proteolysis, then such secondary sites remain available.

Finally, can the current model explain the presence of an efficient ZP block to polyspermy in the absence of ZP2 proteolysis in ZP2-rescue mice? This may be more difficult. Monospermic fertilization cannot simply be attributed to a fast plasma membrane block, since in this case one would expect the accumulation of sperm in the perivitelline space between the ZP and the egg plasma membrane. Rankin et al. clearly show that there are very few perivitelline sperm. The current model would require that low levels of ZP2 proteolysis are present, possibly along the inner face of the ZP abutting the perivitelline space. A narrow zone of proteolysis would, according to the current model, generate a sperm-impermeable layer and yet be difficult to detect by gel electrophoresis. In this regard, ZP2 is a member of a set of reproductive genes that are evolving at a rapid rate and interacting genes may exhibit positive Darwinian selection (Swanson and Vacquier, 2002). Mouse and human ZP2 are orthologous proteins but not highly conserved. Thus, human ZP2 may be a poor substrate for the mouse ZP2 protease due, possibly, to sequence differences in the vicinity of the assumed ZP2 protease cleavage site. Fortunately, this can be readily tested by demonstrating the ZP2 cleavage site directly, and then producing transgenic rescue animals with a mutant mouse ZP2 that cannot be cleaved by the ZP2 protease. If such animals continue to mount a ZP block to polyspermy, then it may be necessary to revise major aspects of the current model of mammalian fertilization.

Conclusions

The current model of mammalian gamete interaction, and particularly its core elements, were assembled during the previous two decades and account for many (though not all) of the early events of fertilization. The three papers featured here use new experimental systems and question aspects of the current model. Critical assessment in the next years will determine the extent to which the current model must be modified or whether it must be replaced.

Selected Reading

Ensslin, M.A., and Shur, B.D. (2003). Cell *114*, this issue, 405–417.

Evans, J.P., and Florman, H.M. (2002). Nat. Cell Biol. (Suppl.) *4*, S57–S63.

Fukami, K., Yoshida, M., Inoue, T., Kurokawa, M., Fissore, R.A., Yoshida, N., Mikoshiba, K., and Takenawa, T. (2003). J. Cell Biol. *161*, 79–88.

He, Z.Y., Brakebusch, C., Fassler, R., Kreidberg, J.A., Primakoff, P., and Myles, D.G. (2003). Dev. Biol. *254*, 226–237.

Jungnickel, M.K., Marrero, H., Birnbaumer, L., Lemos, J.R., and Florman, H.M. (2001). Nat. Cell Biol. *3*, 499–502.

Minke, B., and Cook, B. (2002). Physiol. Rev. *82*, 429–472.

Primakoff, P., and Myles, D.G. (2002). Science *296*, 2183–2185.

Rankin, T.L., Coleman, J.S., Epifano, O., Hoodbhoy, T., Turner, S.G., Castle, P.E., Lee, E., Gore-Langton, R., and Dean, J. (2003). Dev. Cell *5*, 33–43.

Shemer, G., and Podbilewicz, B. (2003). Bioessays *25*, 672–682.

Singson, A. (2001). Dev. Biol. *230*, 101–109.

Springer, T. (1994). Cell *76*, 301–314.

Swanson, W.J., and Vacquier, V.D. (2002). Nature Rev. Genetics *3*, 137–144.

Thaler, C.D., and Cardullo, R.A. (1996). J. Biol. Chem. *271*, 23289–23297.

Wassarman, P.M., Jovine, L., and Litscher, E.S. (2001). Nat. Cell Biol. *3*, E59–E64.

Xu, X.-Z.S., and Sternberg, P.W. (2003). Cell *114*, 285–297.